AD NUMBER

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PURIFICATION OF INFLUENZA VIRUS POLYPEPTIDE ANTIGENS AND STUDIES OF THEIR IMMUNOGENICITY AND TOXICITY

ANNUAL PROGRESS REPORT

by

Edwin D. Kilbourne, M. D.
Doris J. Bucher, Ph. D.

September, 1976

(For the period 31 July 1975 - 1 August 1976)

Supported by
U. S. Army Medical Research and Development Command
Office of the Surgeon General, Washington, D. C. 20315

Contract No. DADA17-69-C-9137

Mount Sinai School of Medicine
of the City University of New York 10029

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SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER

2. GOVT ACCESSION NO.

3. RECIPIENT'S CATALOG NUMBER

4. TITLE (and Subtitle)
PURIFICATION OF INFLUENZA VIRUS POLYPEPTIDE ANTIGENS AND STUDIES OF THEIR IMMUNOGENICITY AND TOXICITY.

5. TYPE OF REPORT & PERIOD COVERED
Annual Report
July 31 1975 - Aug 1 1976

6. PERFORMING ORG. REPORT NUMBER

7. AUTHOR(s)
Edwin D. Kilbourne and Doris J. Bucher

8. CONTRACT OR GRANT NUMBER(s)
DADA17-69-C-9137

9. PERFORMING ORGANIZATION NAME AND ADDRESS
Mount Sinai School of Medicine of the City University of New York, Fifth Avenue and 100th Street, New York, New York 10029

10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS

11. CONTROLLING OFFICE NAME AND ADDRESS
US Army Medical Research and Development Command, Washington, D.C. 20314

12. REPORT DATE
Sept 1976

13. NUMBER OF PAGES

14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)
Annual progress rept. 31 Jul 75 - 1 Aug 76

15. SECURITY CLASS. (of this report)

15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)
Distribution limited to US Government agencies only; proprietary information; June 1972. Other requests for this document must be referred to Commanding General, US Army Medical Research and Development Command. Attn: SGRD-RP

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)
12 34p.

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)
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DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

404463 LB

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Vaccine trials have compared the immune response of a neuraminidase-specific vaccine employing the recombinant X-38 (Heq1N2) with that of a conventional bispecific vaccine, X-37 (H3N2). A superior antibody response to neuraminidase was observed when the enzyme was coupled with the "irrelevant" hemagglutinin in the X-38 vaccine as compared with the conventional vaccine. An unexpected finding was the induction of anti-H3 heterotypic antibody by the X-38 vaccine possessing the "irrelevant" hemagglutinin. However, these antibodies are apparently non-neutralizing.

A neuraminidase-specific vaccine for swine influenza (X-54a) has been produced as an antigenic hybrid containing the swine influenza virus neuraminidase combined with an "irrelevant" hemagglutinin (Heq1 N1sw).

Preliminary trials of immunization of man with isolated neuraminidase antigen revealed: 1) no toxicity, 2) adequate immunogenicity and 3) contamination of the preparation with viral hemagglutinin.

Techniques for viral protein purification have been improved and have shown that hemagglutinin as a dimer is immunogenic.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Science - National Research Council.

SUMMARY

Much of our report deals with efforts directed towards the continued development of an "infection-permissive" neuraminidase-specific vaccine. Such a vaccine may be produced either by tailor-making a recombinant virus containing the neuraminidase of the current subtype of influenza virus coupled with an irrelevant hemagglutinin or by purification of the neuraminidase directly from the vaccine.

Vaccine trials have been conducted which compare the immune response of a neuraminidase-specific vaccine employing the recombinant X-38 (HeqlN2) with that of a conventional bispecific vaccine, X-37 (H3N2). Three groups of college students received either conventional A/England/42/72 (H3N2) vaccine (X-37), neuraminidase monospecific vaccine (HeqlN2) (X-38), or a placebo injection. An interesting finding was the superior antibody response to neuraminidase observed when the enzyme was coupled with the "irrelevant" hemagglutinin in the X-38 vaccine as compared with the X-37 vaccine. The homotypic hemagglutination inhibition response to vaccine hemagglutinin occurred with almost the same frequency among vaccinees receiving either X-37 or X-38 vaccines, with a lesser degree of response for X-38. An unexpected finding was the significant induction of anti-H3 antibody by the X-38 vaccine possessing the "irrelevant" hemagglutinin. However, unlike the usual antibody response to hemagglutinin, these antibodies are apparently non-neutralizing.

Neuraminidase-specific immunization for protection against swine influenza will also be possible using an antigenic hybrid (X-54a) containing the swine influenza virus neuraminidase combined with an "irrelevant" hemagglutinin (Heql Nlsw). This virus is

already under experimental production as a vaccine by Lederle Laboratories for use in field trials.

Preliminary results are reported for a neuraminidase-specific vaccine utilizing the enzyme purified from a commercial lot of vaccine. Following disruption of the virus, the neuraminidase was purified by adsorption and elution from an affinity column. The major modification required for production of the neuraminidase for vaccine purposes was the substitution of Tween 80 for Triton X-100 as a stabilizing agent, permitting use in human subjects. An extra benefit of the preparation procedure was a significant lowering of contaminating endotoxin. Immune response to the purified neuraminidase was good in human subjects when the dose was adjusted to the equivalent enzymatic activity of the starting vaccine preparation. However, considerable hemagglutinin contaminated the enzyme preparation as could be seen by the hemagglutination inhibition titers, particularly at the higher doses. Future neuraminidase vaccine preparations will require harsher conditions for disruption of the virus and the use of recombinant viruses containing detergent sensitive hemagglutinins.

Chromatographic isolation of the hemagglutinin, neuraminidase and other viral polypeptides has continued with the aim of producing sufficient quantities of purified materials for studies of the immunogenicity and biochemistry of these proteins. A cross-linked agarose media permits chromatography of larger quantities of viral protein at the first step of gel filtration. A new chromatographic technique has been developed for the removal of the detergent SDS from protein preparations and their subsequent renaturation without the significant losses of material incurred by earlier techniques.

One difficulty of the SDS gel filtration has been the lack of immunogenicity of the purified hemagglutinin polypeptides. Our chromatographically purified hemagglutinin polypeptides as HA_1, HA_2 or as a mixture of HA and NP from SDS gel filtration have been remarkably non-immunogenic. We are now able to achieve a good immune response with a hemagglutinin fraction which elutes from SDS gel filtration apparently as a dimer when the starting viral preparation is disrupted with a low level of SDS to protein.

Our pyrogenicity studies with Dr. Atkins are underway. Commercial vaccine preparations and our own sucrose gradient viral preparations have been examined and both are remarkable for their low level of pyrogenic response in rabbits. Preparations of swine flu vaccine are also being examined with the aim of relating the pyrogenic response in rabbits with reactogenicity in humans.

Table of Contents

Comparative efficacy of neuraminidase-specific and conventional influenza virus vaccines in the induction of anti-neuraminidase antibody in man. (Preliminary findings were summarized in progress report for 1974-75). (J. Infectious Diseases in press) (E. D. Kilbourne)

Groups of college students (30-39 per group) received either conventional A/England/42/72 (H3N2) vaccine (X-37), or an antigenically hybrid (Heq1N2) vaccine (X-38) containing the same neuraminidase (Figure 1), and effectively neuraminidase-monospecific, or a placebo injection. The vaccines contained 798 and 643 CCA units respectively per dose and equivalent immunogenic units of N2 as defined in antigenic extinction tests in rabbits (Table 1). All subjects had pre-immunization anti-N2 antibody and mean initial titers were comparable in both vaccine groups (1:56). Homotypic hemagglutination-inhibition (HI) response to vaccine hemagglutinin occurred with a frequency of 72% in X-37 and 77% in X-38 vaccinees but was less in magnitude in the X-38 than in the X-37 group (Table 2). Significant anti-N2 (NI) response was observed in 25% of X-37 and 69% of X-38 vaccinees (Table 2) - an unexpected finding. Mean NI response was 2 fold greater in the X-38 group. Another unanticipated finding was the occurrence of heterotypic HI response (i.e., versus H3) in 56% of those receiving X-38 vaccine (Table 3). In preliminary plaque inhibition titrations this heterotypic antibody did not have neutralizing activity. Testing of NI response with earlier neuraminidase antigens demonstrated "original antigenic sin" from earlier priming involving in one instance also the N1 neuraminidase (Figure 2). The superiority of the "neuraminidase-specific" X-38 (Heq1N2) vaccine as an immunogen for anti-neuraminidase antibody may reflect different processing of the N2 neuraminidase when it is associated with hemagglutinin antigen to which the study population is inexperienced. (see below)

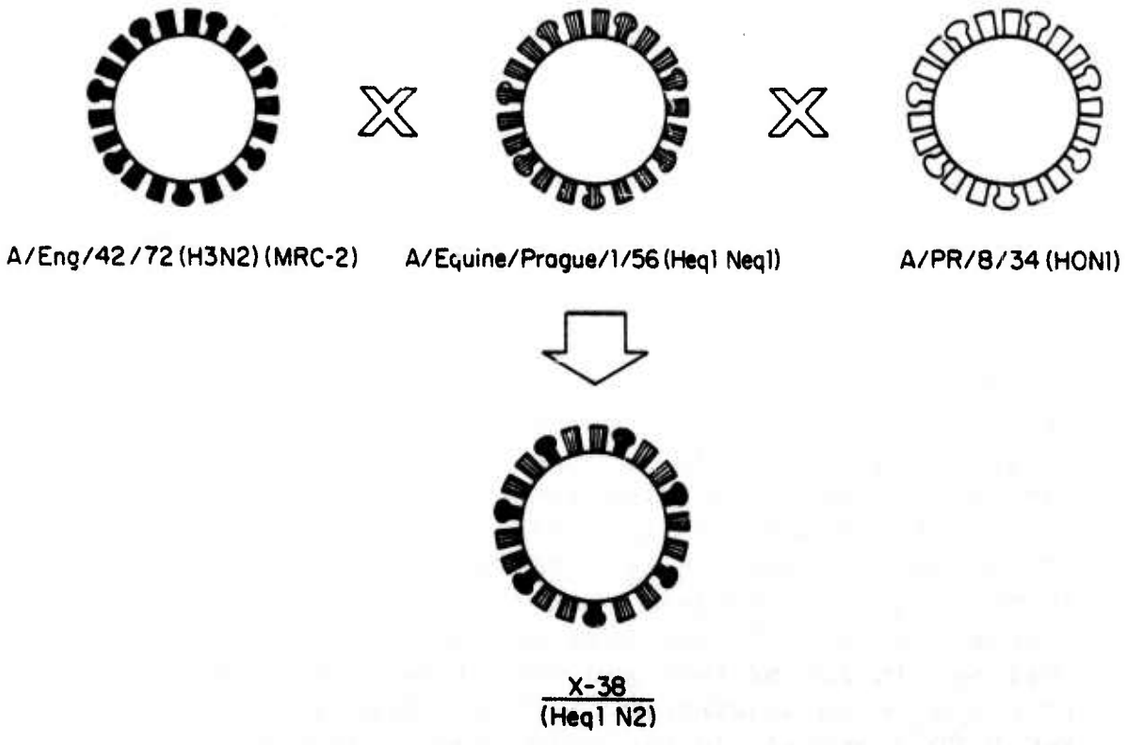

Figure 1. Derivation of antigenically hybrid recombinant virus (X-38) employed as neuraminidase-specific vaccine. Following mixed infection of the chick embryo allantoic sac with the three parental viruses shown, recombinant X-38, bearing Heq1 hemagglutinin and $N2_{Eng}$ neuraminidase, was isolated from the mixed yield by passage with antisera suppressive to viral surface antigens H3, H0, Neq1 and N1. A/PR/8/34(HON1) did not donate surface antigens to X-38 but presumably provided unidentified genes (not shown) associated with high virus yield in the chick embryo. X-37 was derived in the same experiment as a virus serotypically like A/Eng/42/72 and with high yield characteristics.

Table 1. Characterization of vaccines

	Activity		Immunogenic units*	
Vaccine	Hemagglutinin	Neuraminidase	Hemagglutinin	Neuraminidase
X-37 (H3N2)	798**	12.5***	1280	640
X-38 (HeqlN2)	643	9.1	640	640

* per ml. of vaccine - antigenic extinction titrations in rabbits

** chick cell agglutinating units/ml.

*** n moles/ml/min. of N-acetyl neuraminic acid released from fetuin substrate

Table 2. Serologic response to H3N2 and Heq1N2 vaccines

Vaccine	No. of Subjects	Homotypic HI response No.	Homotypic HI response Mean	NI response No.	NI response Mean[4]
None	29	0	0	0	0
H3N2	36	26 (72%)[1]	4.3X	9 (25%)[3]	2X
Heq1N2	39	30 (77%)[2]	2.6X	27 (69%)[3]	4X

[1] vs. H3Neq1 virus
[2] vs. Heq1Neq1 virus
[3] vs. $HO_{PR8}N2_{England}$ virus
[4] mean fold increase in NI antibody

Table 3. Heterotypic HI response to Heq1N2 vaccine (induction of anti-H3 antibody)

Vaccine	No. of subjects	Homotypic HI response		Heterotypic HI response	
		No.	Mean	No.	Mean
None	29	0	0	0	0
H3N2	36	26 (72%)	4.3X	0	0
Heq1N2	39	30 (77%)	2.6X	22 (56%)	2.7X

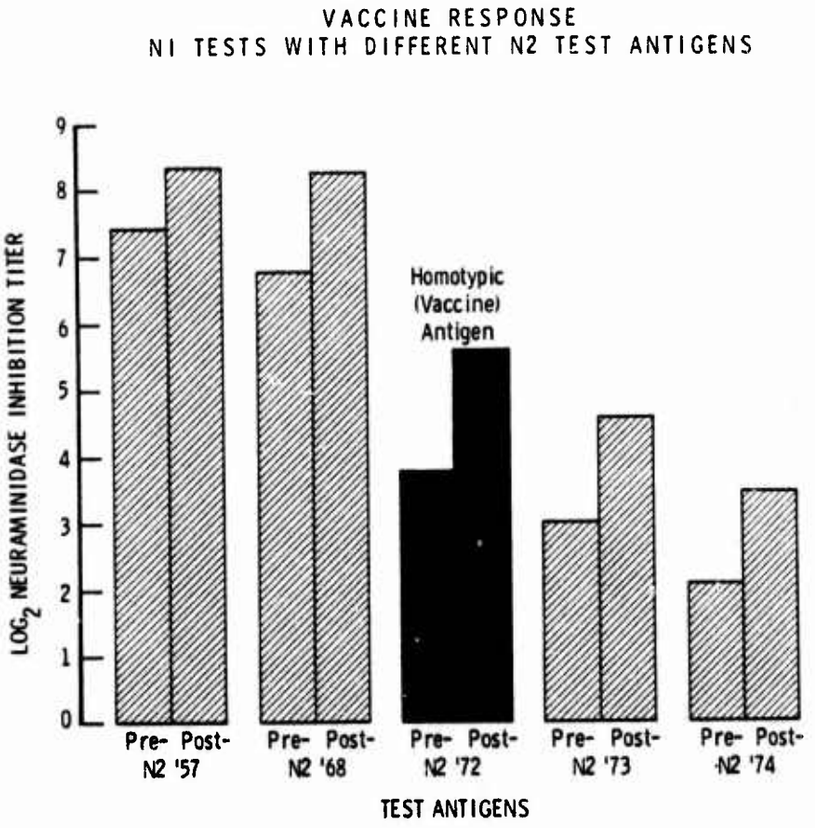

Figure 2. Magnitude of anti-N2 antibody increase in subjects immunized with X-37 (H3N2) (same population as in Figure 2). Maximum fold increases in titer are demonstrated with the antigen homotypic to immunization (N2'72). However, absolute increase is greater to earlier N2 antigens, illustrating "original antigenic sin" or heterospecific anamnestic response. (see text discussion)

On the basis of an earlier study in human subjects (1) it was anticipated that the administration of influenza viral neuraminidase in an antigenically hybrid virus would prove immunogenic for antineuraminidase antibody. Two unexpected findings in the present comparison of an experimental vaccine of conventional bispecific (H3N2) composition with a new antigenic hybrid (Heq1N2) containing equal amounts of N2 antigens were: 1) the superior potency of the hybrid vaccine in engendering antineuraminidase response and 2) induction of heterospecific (anti-H3) antibody by the presumably neutral or irrelevant Heq1 hemagglutinin of the recombinant hybrid vaccine strain.

In the earlier study, comparison of antineuraminidase antibody responses with those with a vaccine of conventional composition had not been made and all subjects had been sero-negative for anti-H3 HI antibody prior to immunization, and pre-immunization anti-NA antibody either was not demonstrable or was present only in low concentration. Thus, lack of a contemporaneously administered, matched conventional vaccine and the utilization of a relatively unprimed population, probably precluded demonstration either of the hybrid vaccine's superiority or heterotypic response in the earlier study (1). It is notable that in studies of another Heq1-containing hybrid vaccine, X-42, (Meiklejohn and Eickhoff, personal communication) HI cross-reactions detected with test virus bearing the N2 neuraminidase (H3N2) were virtually eliminated when a hemagglutinin-monospecific test antigen was employed. Furthermore, HI heterotypic responses were absent in children immunized with the same vaccine (X-42), probably in relation to the lesser priming of that population with the H3 antigen (Ogra, et al., personal communication).

Of critical importance to the interpretation of protection studies with antigenic hybrids containing the Heq1 HA, are the preliminary data suggesting that the heterotypic antibody induced is non-neutralizing to H3-containing potential challenge viruses. Thus, in accord with the assumptions on which the strategy of immunization is based, any protection induced can

be interpreted as probably the consequence of increase in antineuraminidase antibody alone. That is, significant increase in neutralizing antibody might reduce the probability of successful challenge infection essential for the definitive immunizing step required in the immunization strategem of neuraminidase-specific vaccination. Therefore, it is encouraging that preliminary evidence indicates that the heterotypic antibody that is formed is non-neutralizing. Efforts to select a variant of Heq1 non-cross-reactive with H3 antigen have been frustrated thus far by the absence of plaque neutralization of Heq1Neq1 virus by anti-H3 antiserum. This approach is reasonable in view of evidence that influenza viral hemagglutinin contains at least two antigenic determinants that are either strain specific (homotypic) or cross-reactive (heterotypic), that are subject to independent variation (2, 3). Indeed, elimination of cross-reactivity of an H3 strain with Heq2 has been obtained by selective passage of H3N2 virus with Heq2 antiserum, (Schulman, J. L., unpublished data) although in that instance the selected virus was still heterotypically immunogenic. Complete elimination of hemagglutinin-mediated cross-reactivity obviously can be achieved by the use of isolated purified viral neuraminidase as antigen, but studies of the immunogenicity of such preparations in man are only in their inception. In the meantime, the continued exploratory use of antigenic hybrids as neuraminidase-specific immunogens should provide useful information.

On the basis of previous study of age related influenza viral neuraminidase antibody patterns of man (4), and recognition of distinct antigenic neuraminidase variants, even within the same viral subtype (5), and recall of 1964 neuraminidase antibodies by 1968 influenza A virus immunization (6), it was not surprising to find heterotypic anamnestic response to the introduction of neuraminidase antigen. Response in terms of number of subjects experiencing increase in neuraminidase antibody arbitrarily defined as significant was best demonstrated with homotypic N2/72 antigen, but the amount of antibody formed was

greater to N2 antigens of earlier prevalence - those with whom the population had had their initial experience. These observations reemphasize the question already raised on the basis of heterotypic anamnestic response to the hemagglutinin antigen of the wisdom of vaccine strain changes in response to every minor antigenic variation of the virus.

The immunogenic superiority of N2 antigen when administered in an antigenically hybrid virus containing hemagglutinin irrelevant to human experience was unexpected. It must be emphasized that X-37 and X-38 vaccines were matched vaccines in every way. They were produced simultaneously by the same manufacturer by the same process and were standardized on the basis of antigenic extinction titrations to equivalent immunogenicity in experimental animals. Furthermore, the animal studies furnish direct evidence that when given at equal dosage the two vaccines induced comparable levels of NI antibody in primary immunization. Although not necessarily related to immunogenicity, the neuraminidase activity of the vaccines was comparable, with the antigenic hybrid, X-38, actually having less activity than the bispecific X-37 vaccine that is representative of present conventional vaccines. These observations are reinforced by those from study of the comparably matched vaccines, X-41 and X-42 (Ogra, et al., personal communication), in which the antigenic hybrid, X-42, also induced greater frequency and magnitude of response to the neuraminidase antigen.

Thus, explanation for the phenomenon must be sought in differing immunologic response to presentation of the neuraminidase antigen in association with different hemagglutinin antigens. In subjects primed with H3 antigen, as was the case in the present study, the processing of vaccine viral particles is probably different. On the basis of immunologic memory, H3N2 viral particles may be preferentially directed to H3 memory cells (presumably B lymphocytes [3]), compared to HeqlN2 particles for which the subjects are primed principally with respect to N2. Although the mechanisms and details of such antigen processing can only be

speculated upon, it is not unreasonable that the presence of a memory system for H3 might competitively reduce the opportunity for N2 to unite definitively with B or T cell receptors for the induction of anti-N2 response, when the H3 and N2 antigens are borne by the same particle.

In contrast, the absence in human subjects of an expanded population of memory cells for the alien antigen Heq1 might result in competition in which N2 antigen is preferentially absorbed by N2 memory cells in subjects given Heq1N2 vaccine, while response to the associated Heq1 antigen that reaches antibody forming cells is primary. This formulation ignores the demonstrated cross-reactivity of Heq1 and H3 antigens, but it can be assumed that the affinity of Heq1 for H3 memory cells would be less than in homotypic response. In fact, B cell-specific adoptive immunization experiments with purified hemagglutinin antigen of cross-reactive influenza viruses of other subtypes (H0 and H1) confirm a greater magnitude of homotypic than heterotypic response when primed animals are initially stimulated with the cross-reactive antigen (7).

Studies of cell mediated immunity in influenza are just beginning, and it is clear that they must take cognizance of possible differences in the processing of hemagglutinin and neuraminidase antigens as well as differing modulation of the immune response dependent upon varying backgrounds of immunizing experience and relatedness of variants of either antigen. All these factors muse be considered as experiments with neuraminidase-specific immunization are continued, and especially as this procedure is employed as a prelude to administration of live virus vaccines.

<u>Preparation of an antigenic hybrid for use in neuraminidase-specific immunization against swine influenza</u>

With the appearance of any new influenza virus variant it has been our custom to prepare an antigenic hybrid that incorporates the Heq1 hemagglutinin and the neuraminidase of the new strain. Such recombination

assumes special significance in the case of the Ft. Dix A/sw/NJ/76 virus to which younger members of the population lack antibody. Therefore an unprecedented opportunity is provided for the primary antigenic stimulation of humans by monospecific immunization with a neuraminidase to the swine virus. Recombination as indicated in Figure 3 was carried out employing X-53a, the high yielding A/sw/NJ/11/76 recombinant used in vaccine production and A/Prague/1/56 (HeqlNeql) the usual hemagglutinin donor. Derivation of the new hybrid X-54a is shown in Figure 3. This virus is now in experimental production by Lederle Laboratories for use in projected field trials that may receive NIAID sponsorship.

Figure 3.

Derivation of X-54a antigenically hybrid recombinant [$Heq1N1_{sw(NJ)}$]

Post-recombination passage	Recombination
	X-53a X A/Prague/1/56 (Heq1Neq1)
1	10^{-1} A/NJ/11/76 and H_{hk}Neq1 ab.
	HA+
2	10^{-7} A/NJ/11/76 ab.
	HA+
3	10^{-8} A/NJ/11/76 ab.
	HA^+ 4/6 eggs
4	10^{-5}
	E-3515* (provisional seed virus)
5	10^{-5}
	HA^+
6	10^{-5}
	HA^+
7	10^{-5}
	E-3526 (provisional seed virus) HA by tube dil. = 1:4096
8	$10^{-9.5}$ (cloning)
	1/10 eggs HA^+*
9	10^{-5}
	HA^+ (20 hr. passage)
10	10^{-5}
	E-3531* (definitive seed virus) A/Prague/1/56-NJ/11/76 ($Heq1N1_{sw}$) X-54a

*verified as Heq1N1sw by HI and NI

Neuraminidase vaccine of isolated viral protein
(with Douglas and Cerini, Lederle Laboratory)

Preparation of the vaccine

Neuraminidase can be purified from influenza virus or vaccine by adsorption and elution from an affinity column bearing an inhibitor to neuraminidase, aminophenol oxamic acid, as originally devised by Cuatrecasas and Illiano (1971) (8) and subsequently modified for purification of the viral neuraminidase ([9] and earlier progress reports). There was an average increase of 10.6 fold in specific activity when X-38 vaccine was the starting preparation.

The availability of the purified neuraminidase from influenza vaccine in milligram quantities permits the testing of the neuraminidase-specific infection-permissive vaccine using the neuraminidase as the immunizing agent. The concept of the neuraminidase-specific vaccine has been developed by E. D. Kilbourne and associates, 1972 (10), and Couch and associates, 1974 (1). Use of the purified neuraminidase free of hemagglutinin would eliminate the problem of cross-reactivity of the "irrelevant" hemagglutinin in the recombinants originally proposed as the source for a neuraminidase-specific vaccine. An additional advantage of the use of the purified neuraminidase as the vaccine would be the probable lowered reactogenicity of such a preparation. Subunit vaccines prepared by disruption of viral particles with sodium deoxycholate have been shown to be non-pyrogenic (11).

The feasibility of preparing such a vaccine under sterile conditions for clinical trials was tested in our laboratory. The starting preparation was a commercial vaccine of MRC-11, a high yielding recombinant of "London flu" virus. An affinity column was prepared by the usual procedure under sterile conditions (8, 9). All buffers and glassware were sterilized. The purification technique was that generally employed for the preparation of neuraminidase from virus or vaccine lots. The one major change required for the preparation of vaccine suitable for

administration to human subjects was the substitution of Tween 80 (considered an "injectable" by the FDA) for that of Triton X-100. Both compounds are non-ionic detergents and substitution of the Tween 80 for Triton X-100 was not a major barrier. Triton X-100 is used in the purification of neuraminidase as a stabilizing agent rather than as a disrupting agent. Generally Triton X-100 is added at a level of 0.1 - 0.2% to all buffers employed in adsorption and elution and to a level of 10% to the viral preparation after disruption with 1% SDS. If these same conditions were maintained but Tween 80 substituted for Triton X-100, the recovery of neuraminidase activity from the affinity column was only 1.7%. If the level of Tween 80 was elevated to 0.5% in the buffers and to 20% after the SDS disruption step, then the recovery of neuraminidase from the affinity column was 25%. Reduction of the anionic detergent SDS to a level of 0.5% (from 1%), resulted in elution of the enzyme from the column with yields of 32 - 49%. The final preparation of neuraminidase employed for vaccine in clinical trials represented a yield of 32% and had an increase in specific activity of 3.7 fold. Additional denaturation occurred with the "packaging" of the neuraminidase. The neuraminidase vaccine in the vials had a specific activity only two fold higher than the starting vaccine preparation: a stabilizing agent may be necessary for preservation of enzyme activity. This low increase in specific activity was apparently due to a substantial amount of contamination with nucleoprotein as visualized on polyacrylamide gels. A total of five vaccine batches were prepared. Two batches had a substantial quantity of hemagglutinin remaining as seen on polyacrylamide gels; however, these preparations were not used in the vaccine trial. This contamination was apparently due to the use of excessively mild conditions for disruption of the viral particles with SDS; these preparations had been disrupted with SDS at 4^{o}, cleaner preparations were obtained when the disruption with SDS was performed at room temperature.

An additional complication in the procedure was the presence of 0.25% Tween 80 in the starting vaccine preparation. Since the nonionic detergent probably has a protective effect countering the disruption of the viral particles with SDS and resulting in contamination of other viral proteins, future neuraminidade vaccines will be prepared after centrifuging the viral particles from the vaccine solution to eliminate most of the Tween 80 before the SDS disruption step.

An additional concern had been the contamination of the neuraminidase with amino-phenol oxamic acid from the affinity column. A certain amount of "bleed" does occur with the first few cycles of a new column. Therefore a series of three trial runs was conducted before the actual vaccine batches were adsorbed and eluted from the column. The minimal level of detection was 4 nanograms/ml. The first of the five vaccine batches had a level of 40 nanograms/ml. However, the final preparation used in the clinical trials contained no detectable amino-phenol oxamic acid.

An additional benefit of the chromatographic preparation of the neuraminidase was the substantial lowering of endotoxin contamination. Whereas the original vaccine preparation required a dilution 1:200 to reach an endpoint in the limulus assay, the purified neuraminidase had an endpoint of 1:10.

Preliminary studies of antigenicity of neuraminidase vaccine

At the dose level of 0.1 ml., adjusted to the volume of a normal vaccine dose on the basis of neuraminidase activity, three out of the four subjects responded with 8 to 25 fold increases in antibody level as measured by neuraminidase inhibition with an average increase of 17 fold (see Table 4). However, three out of the four subjects responded with an increase in antibody to hemagglutinin, one at a 2 fold level, two at a four fold increase, representing an average increase of 3 fold.

Table 4. Serologic response to purified neuraminidase

Vaccine dose	No. of subjects	HI response[1]		NI response[2]	
		No.	Mean fold increase	No.	Mean fold increase
0.1 ml	4	3	3	3	17
0.5-2.0 ml	6	6	19	6	13

[1] versus H3 (Pt. Chalmers)/Neq1 recombinant
[2] versus Heq1/N2 (Pt. Chalmers) recombinant

At dose levels of five to 20 fold higher levels of the neuraminidase vaccine, the six subjects had 4 to 32 fold increases in neuraminidase inhibiting antibodies, representing an average increase of 13 fold. At these high doses, the hemagglutinin contamination became quite obvious with antibody responses measured as hemagglutination inhibition ranging from two to 64 fold, with an average increase of 19 fold.

Thus it can be seen that good immunologic response to the neuraminidase was achieved at 0.1 ml., a dose equivalent to the neuraminidase activity in the original MRC-11 vaccine. Therefore, the purified neuraminidase possesses good immunogenicity and it is apparently valid to adjust the dose to equivalence with the neuraminidase activity of the intact virus vaccine. Higher doses of the neuraminidase vaccine did not result in any appreciable increase in antibodies to neuraminidase but did produce a significant antibody response to the hemagglutinin which apparently contaminated this preparation. Modifications can be introduced at several points to eliminate such contamination in the future.

More severe conditions for disruption of the viral particles at the point of addition of SDS may be of value. These conditions would include elevation of the SDS level to 1% and the elimination of the 0.25% Tween 80 from the vaccine preparation before SDS disruption.

Additionally, tailor-made recombinants may be used to eliminate contamination with hemagglutinin. These would include the use of hemagglutinins in recombinants which are SDS-sensitive, such as the hemagglutinin from WS or certain H2N2 strains (Webster and Laver, 1966) and/or the use of "irrelevant" hemagglutinin which would have low cross-reactivity if not completely removed.

In summary, these preliminary results indicate that the purified neuraminidase possesses good immunogenicity and the feasibility of the preparation of such a vaccine has been demonstrated. However, future vaccine preparations will require additional testing to insure that all contaminating hemagglutinin has been removed.

Chromatographic isolation of the viral polypeptides

Modifications of the chromatographic system have included the use of Sepharose 6B-CL for the first stage of separation of the HA-NP fraction. This gel filtration material is considerably more rigid due to the introduction of cross-linking groups in the gel. Since the gel material is more rigid, higher concentrations of protein can be applied permitting the separation of very large quantities of the purified proteins. The sample is applied in 20 ml. quantities of very high viscosity. Although the Bio Gel A-5m permits finer resolution, it is a very soft gel which tends to compact and shut off the flow rate with viscous samples. The column technique now includes a first stage separation of the very viscous total viral proteins on Sepharose 6B-CL followed by concentration and reapplication of the HA-NP fraction to Bio Gel A-5m permitting fine resolution of this fraction and improving separation of HA-NP from P and M. The HA-NP fraction is then reduced and reapplied to the Bio Gel A-5m solumn to separate the HA_1 from HA_2.

The SDS can now be removed from the protein using a new technique which we have developed which involves the use of an anion exchange gel with 6 M urea. The substitution of anion exchange gel (QAE Sephadex) for DEAE cellulose greatly improves the protein yield from the column. The 6 M urea must be included to insure quantitative recovery. Whereas the DEAE cellulose permitted recovery of 40 - 80% of the viral protein with removal of detergent, the QAE Sephadex permits recovery of 80 - 100% of the total protein. This change in technique permits a very considerable improvement in yield of protein and allows a large savings in material.

Formation of immunogenic soluble hemagglutinin --

Relationship of the substructure of hemagglutinin to immunogenicity

It has become apparent that the immunogenicity is related to the degree of substructure of the hemagglutinin. With the use of a lower ratio of SDS/protein for disruption of the viral particles, a large proportion of the hemagglutinin migrates as a 150,000 component on SDS gel filtration rather than the 75,000 unit -- suggesting a dimeric association of the hemagglutinin. Use of the "dimeric" hemagglutinin as an immunogen for rabbits has resulted in the development of hemagglutination inhibiting (HI) antibodies, whereas previously only a very low HI response could be obtained with HA_1, HA_2 or HA-NP.

The immunogenicity of HA_1 and HA_2 as separated by SDS gel filtration has been shown to be very low (Erickson and Kilbourne, unpublished results). The HA_1 and HA_2 polypeptides were chromatographed to remove SDS and "renatured" according to Weber and Kuter (1971) (12). The HA-NP fraction was similarly treated. Quantities of 100 micrograms were used for initial immunization and the subsequent booster dose. The HI response was 1:40 to 1:80. A similar quantity of "dimeric" hemagglutinin under the same conditions resulted in an HI of 1280 under similar conditions, either with or without prior removal of the SDS.

From the chromatographic elution of the hemagglutinin polypeptide(s) on SDS gel filtration, it is apparent that there are two states of SDS solubilization of the hemagglutinin spike: (1) occurs with release of the immunologically active state probably as a dimer aggregate and (2) additional SDS converts the HA dimer to the monomeric form which is antigenically and biologically inactive. The addition of a reducing agent to the monomeric hemagglutinin converts the glycoprotein to HA_1 plus HA_2. The various proposed states of existence of the hemagglutinin polypeptide(s) in the presence of SDS are

diagrammed in Figure 4. These findings help to explain the anomaly of SDS "resistant" and "sensitive" hemagglutinins and the corresponding association of activity with these preparations (11). Those hemagglutinins which appear "resistant" to SDS probably bind HA poorly and are in the dimeric form, those hemagglutinins which are "sensitive" would readily bind SDS and be converted to the monomeric form which is no longer immunogenic. Thus careful control of detergent level can yield hemagglutinin in the dimeric form which can be acetone or ethanol precipitated from the SDS containing solution and which possesses biologic activity. The detergent/protein ratio is important in determining biologic and immunologic activity.

Evidence from hemolysis of red blood cells would suggest that at least twice as much detergent is present in a similar volume for the monomeric as for the dimeric form. However, the detergent to protein ratio has not been calculated on a molar basis and must be determined for an accurate understanding of detergent/protein interaction.

PROPOSED RELATIONSHIP OF SUBSTRUCTURE OF HEMAGGLUTININ TO IMMUNOGENICITY

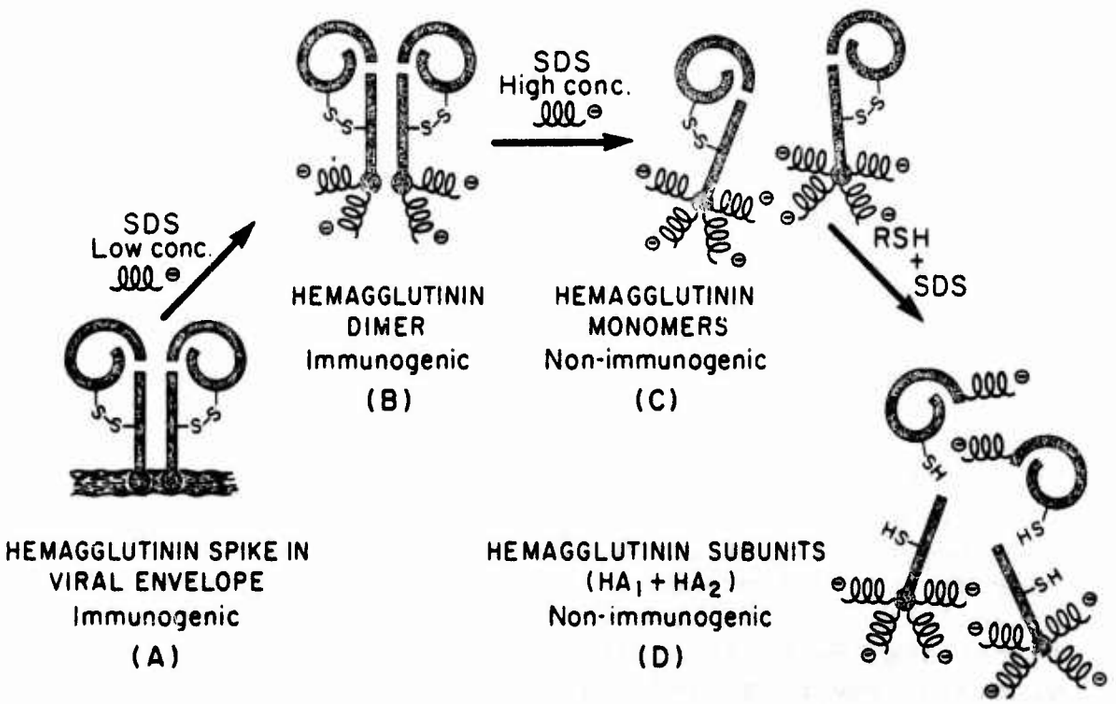

Figure 4.

Pyrogenicity experiments (with Dr. Atkins)

The accompanying high fevers frequently occurring in persons immunized with influenza vaccine continue to be a problem even with the highly purified virus preparations obtained through gradient separation of the virus. This undesirable side effect leads to a low acceptance rate of the vaccine even though the vaccine can be highly effective.

In collaboration with Dr. Atkins we are seeking the pyrogen producing component(s) of the influenza virion. With the identification of this factor, the component could be eliminated and vaccines free of pyrogen inducer could be produced. With the SDS gel filtration technique of separation of the viral components, yielding all the fractions of the virus, it is possible to test each component independently for its pyrogenic effect. It has been previously shown that the release of endogenous pyrogen in rabbits is dependent on the presence of a biologically active hemagglutinin. The pyrogen inducer, however, can be inactivated by UV irradiation even though the hemagglutinin remains active. Thus the pyrogenic factor is not the hemagglutinin but requires its activity to cause the induction of endogenous pyrogen. Subunit vaccines are known to be free of pyrogenic factor and thus far it has not been possible to recombine fractions from the subunit vaccines to reactivate the pyrogenic factor.

Sterile virus preparations free of endotoxin have been produced in the laboratory to examine the pyrogenic effect of intact virus in rabbits. Dr. Atkins has been examining two distinctly different types of influenza viruses -- Aichi and a swine flu recombinant (X-53a). The swine flu recombinant did not produce any pyrogenic response even at undiluted concentrations. The Aichi strain did produce a fever in rabbits, apparently not of the endotoxin type.

Both preparations were quite dilute, 128 and 256 HAU/ml, respectively, and new preparations of higher concentrations are being processed. These preparations were purified on sucrose gradients, dialyzed versus PBS and subsequently tested in rabbits. It is possible that the factor inducing pyrogenicity is unstable and/or dialyzable with exhaustive dialysis.

In addition, Dr. Atkins has been testing several vaccine preparations from the Bureau of Biologics, including swine flu vaccines prepared by four pharmaceutical concerns. One of the swine flu vaccines was shown to be highly reactogenic in humans although relatively free of endotoxin. Currently these preparations are being studied with the aim of determining the degree of correlation of pyrogenicity in rabbits with reactogenicity in humans.

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